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ORIGINAL ARTICLE

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Cyclosporin and tacrolimus do not potentiate oxidative damage in pulmonary epithelial cells

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Abstract Oxidative stress can lead to cellular injury and apoptosis within the pulmonary allograft. We investigated the effects of oxidative damage on the growth and survival of cultured human pulmonary epithelial cells treated with hydrogen peroxide (H_2O_2) in the presence and absence of cyclosporin A (CsA) and tacrolimus. Treatment of A549 cells with 1 mmol/l H_2O_2 for 48 h led to a 39% decrease in cell growth. Treatment with 500 ng/ml CsA for 48 h reduced cell survival by 68%, and treatment with 30 ng/ml tacrolimus reduced cell survival by 32%. The addition of CsA or tacrolimus to cells grown in H_2O_2 did not further diminish cell survival. These studies demonstrated that H_2O_2, CsA, and tacrolimus treatments decrease survival of pulmonary epithelial cells. However, CsA and tacrolimus do not further potentiate H_2O_2-induced toxicity.

Keywords Pulmonary epithelial cells · Cyclosporine · Tacrolimus · Oxidative injury · Hydrogen peroxide

Introduction

Oxidative stress can lead to cellular injury and apoptosis. Oxidative damage sustained by epithelial cells and subsequent apoptosis within the pulmonary allograft has been proposed as a factor in ischemia–reperfusion injury [1, 2, 3], acute rejection [4, 5], and subsequent development of obliterative bronchiolitis (OB) [6]. Alterations in the cell cycle in response to oxidative stress lead to decreased survival and apoptosis in pulmonary epithelial cells [7, 8]. However, the activation of cell-cycle checkpoints that alter cell growth following oxidative injury has also been proposed as a mechanism to facilitate repair of DNA damage; for review see [9].

Immunosuppressive agents alter the cell cycle and cell growth via various mechanisms. Tacrolimus and cyclosporin A (CsA) act through the calcineurin pathway to inhibit interleukin-2 (IL-2) expression. CsA also induces expression of transforming growth factor (TGF)-β in pulmonary epithelial cells, thereby inhibiting their proliferation [10, 11].

Therefore, we investigated the effects of CsA, tacrolimus, and oxidative damage on the growth and survival of cultured human A549 pulmonary adenocarcinoma cells. The effect of CsA and tacrolimus on A549 cell survival in the presence and absence of hydrogen peroxide (H_2O_2) was determined. A549 cells were chosen for these experiments because they have a well-characterized response to H_2O_2-induced oxidative injury [12].

Materials and methods

Cell culture

A549 pulmonary adenocarcinoma cells (ATCC # CCL-185) were grown in Kaighn's modified Ham's F12 medium containing 1.5 g/l sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Life Technologies, Gaithersburg, Md.,

USA) and supplemented with 10% fetal bovine serum (BioFluids, Rockville, Md., USA). Cells were grown at 37°C in a humidified incubator containing 5% CO_2.

CsA, tacrolimus, and hydrogen peroxide treatments

A549 cells were plated at 2.5×10^5 cells/well in 24-well plates. The next day, the cells were treated with 0–500 ng/ml CsA (Calbiochem, San Diego, Calif., USA) or 0–30 ng/ml tacrolimus (Calbiochem). After an additional 48 h trypsin was added and the cells were counted; cell viability was determined by trypan-blue exclusion.

The effect of CsA and tacrolimus on cell survival in the presence of H_2O_2 was also investigated. Initially, the growth of A549 cells in medium containing 0.1–5 mmol/l H_2O_2 for 48 h was determined. Cell survival declined significantly when cells were exposed to 0.25 mmol/l H_2O_2. No cells survived treatment with 5 mmol/l H_2O_2 (data not shown). Based on these results, 1 mmol/l $H_2O_2$was used for further studies.

One day after seeding, cells were treated with 1 mmol/l H_2O_2 in the presence and absence of 0–500 ng/ml CsA or 0–30 ng/ml tacrolimus. After an additional 48 h trypsin was added and the cells were counted. Cell viability was determined by trypan-blue exclusion.

Four independent cultures were used for each culture condition. Using Student's *t*-test, we compared cell survival.

Results

As expected, H_2O_2 led to a dose-dependent decrease in A549 cell survival [12]. Cell survival declined significantly when cells were exposed to 0.25 mmol/l H_2O_2. No cells survived treatment with 5 mmol/l H_2O_2 (data not shown). The effects of the immunosuppressive drugs CsA and tacrolimus on A549 cell survival were also determined. Both drugs decreased cell survival at therapeutic concentrations. CsA had a more pronounced effect on cell survival than tacrolimus. Treatment with 500 ng/ml CsA reduced the number of cells that survived by 68%, when compared with untreated cultures (Fig. 1A). All but the lowest CsA concentration that was tested prevented cell growth. Treatment with 30 ng/ml tacrolimus for 48 h slowed A549 cell growth and reduced the number of surviving cells by 32%, when compared with untreated cells (Fig. 2A).

To investigate whether these immunosuppressive drugs would potentiate oxidative damage we treated A549 cells with 1 mmol/l H_2O_2 in addition to either 0–500 ng/ml CsA or 0–30 ng/ml tacrolimus. The addition of CsA (Fig. 1B) or tacrolimus (Fig. 2B) to cells grown in 1 mmole/l H_2O_2 did not further diminish cell survival. These data suggest that the immunosuppressant-mediated alteration in cell survival does not potentiate oxidative damage.

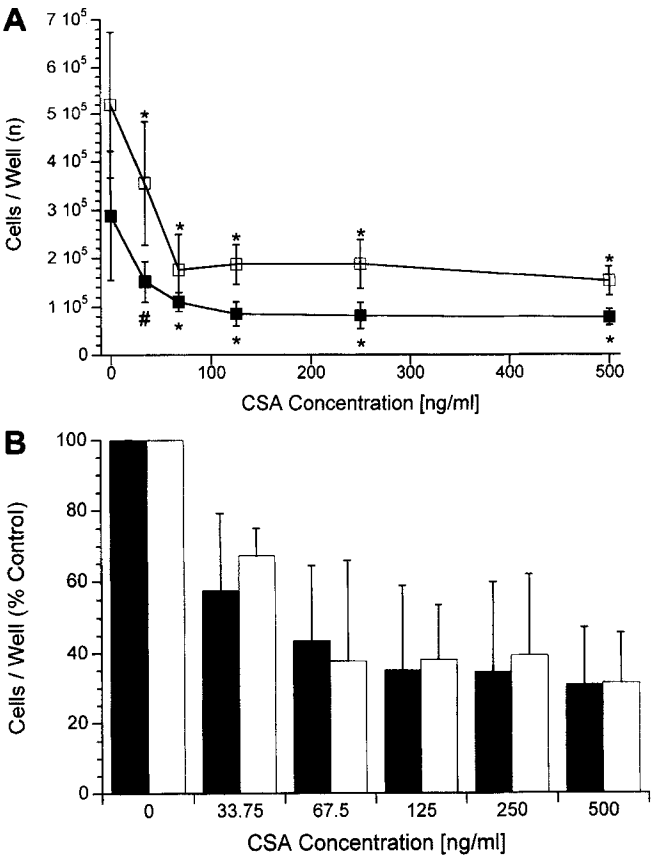

Fig. 1A, B Effect of CsA on cell survival in the presence or absence of H_2O_2. Mean and standard error of the mean of 2–3 independent experiments ($n = 8$–12) is illustrated. **A** Cells treated with CsA in the presence (*solid squares*) or absence (*open squares*) of 1 mmol/l H_2O_2. **B** Cells treated with CsA in the presence (*black bars*) or absence (*white bars*) of 1 mmol/l H_2O_2. The number of surviving cells is expressed as a percentage of surviving untreated cells. $^*P < 0.001$, # $P < 0.05$ cells treated with CsA compared with untreated cells

Discussion

Our data confirm that oxidative injury from H_2O_2 treatment decreases survival of A549 cells. Jarvinen et al [12] also showed that resistance to H_2O_2-induced injury in these pulmonary epithelial cells is correlated with more robust glutathione-mediated anti-oxidant mechanisms. Even though both CsA and tacrolimus decreased survival of A549 cells, these drugs did not potentiate the oxidative injury induced by H_2O_2.

A549 cells were chosen for these studies because they have a well-defined response to oxidative injury [12]. However, our data must be interpreted with caution, since A549 cells may not entirely reflect normal pulmonary epithelial response to injury. In addition, A549 cells have recently been shown to contain subpopulations of cells that may have different responses to drugs and oxidative injury [13].

Epithelial cell damage has been proposed as a crucial factor in the development and evolution of OB. Oxidative injury can damage epithelial cells, leading to cell death and thereby creating an inflammatory milieu.

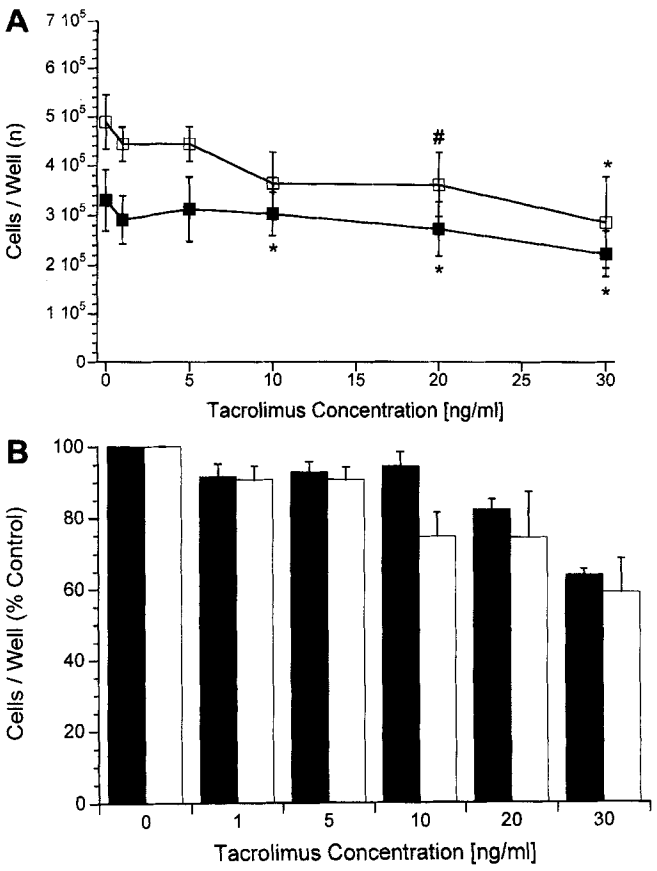

Fig. 2A, B Effect of tacrolimus on cell survival in the presence or absence of H_2O_2. Mean and standard error of the mean of 2–3 independent experiments ($n = 8$–12) is illustrated. **A** Cells treated with tacrolimus in the presence (*solid squares*) or absence (*open squares*) of 1 mmol/l H_2O_2. **B** Cells treated with tacrolimus in the presence (*black bars*) or absence (*white bars*) of 1 mmol/l H_2O_2. The number of surviving cells is expressed as a percentage of surviving untreated cells. $*P < 0.001$, $\#\ P < 0.05$ cells treated with tacrolimus compared with untreated cells

Studies of bronchoalveolar lavage (BAL) fluid obtained from patients with OB showed that such fluid contains increased numbers of inflammatory cells and cytokines [14]. In addition, there may be diminished levels of protective anti-oxidants in this BAL fluid [14].

CsA affects pulmonary epithelial cell proliferation by stimulating TGF-β production [10]. This mechanism has been implicated in the fibrosis that accompanies the development of OB. TGF-β also leads to altered cell growth and, potentially, to defective repair of injured pulmonary epithelium. Zhang et al [10] found that CsA, at high therapeutic concentrations, decreased radiolabeled thymidine uptake into primary airway epithelial cells, while tacrolimus had no effect on DNA synthesis. Our data confirm that CsA leads to decreased survival of pulmonary epithelial cells, but therapeutic tacrolimus concentrations also decreased cell growth. These results may reflect differences in the cells studied or the techniques used to measure cell growth and survival. The differential responses of A549 cells to therapeutic concentrations of CsA and tacrolimus may be related to differences in expression of growth regulators such as TGF-β [10] or p21 [11].

Our data suggest that CsA and tacrolimus do not induce further reduction in cell survival in pulmonary epithelial cells subjected to oxidative injury. Oxidative damage induced by H_2O_2 or hyperoxia causes growth arrest of pulmonary epithelial cells [7, 12]. This process is mediated by several mechanisms, including the induction of the cell cycle regulator p21 [7, 8]. Several authors have proposed that growth arrest is a protective strategy in the face of oxidative damage; for a review see [9]. The alteration in cell growth induced by CsA and tacrolimus could also provide protection against further injury by oxidative stress. The finding that CsA and tacrolimus do not potentiate the oxidative injuries that are commonplace in the pulmonary allograft is reassuring. Ultimately, reduction of oxidative damage will help to protect the pulmonary allograft during ischemia, reperfusion, infections, and rejection.

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